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(71) Applicant *(for all designated States except US)*: **SOCIETE DES PRODUITS NESTLE S.A.** [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LALOI, Maryse** [FR/CH]; Avenue du Grammont, 4, CH-1007 Lausanne (CH). **Mc CARTHY, James** [US/FR]; 298, Vallée de Vautruchot, F-37210 Noizay (FR). **BUCHELI, Peter** [CH/CN]; Building 4, Apart. 2D, Shanghai Racquets Club, jin Feng Road, Minhang District, Shanghai 201107 (CN).

(74) Agent: **PATE, Fred**; Avenue Nestlé 55, CH-1800 Vevey (CH).

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(54) Title: NOVEL CARBOXYPEPTIDASE OF COCOA

(57) Abstract: The present invention relates to a novel carboxypeptidase gene and the polypeptide encoded thereby. In particular, the present invention relates to the use of the present carboxypeptidase in the manufacture of cocoa flavour and/or chocolate.

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Novel carboxypeptidase of cocoa

The present invention relates to a novel carboxypeptidase gene and the polypeptide encoded thereby. In particular, the present invention relates to the use of the present carboxypeptidase in the manufacture of cocoa flavour and/or chocolate.

It is known that in processing cacao beans the generation of the typical cocoa flavour requires two steps – a fermentation step, which includes air-drying of the fermented material and a roasting step.

During fermentation two major activities may be observed. First, the pulp surrounding the beans is degraded by micro-organisms with the sugars contained in the pulp being largely transformed to acids, especially acetic acid (Quesnel et al., J. Sci. Food. Agric. **16** (1965), 441-447; Ostovar and Keeney, J. Food. Sci. **39** (1973), 611-617). The acids then slowly diffuse into the beans and eventually cause an acidification of the cellular material. Second, fermentation also results in a release of peptides exhibiting differing sizes and a generation of a high level of hydrophobic free amino acids. This latter finding led to the hypothesis that proteolysis occurring during the fermentation step is not due to a random protein hydrolysis but seems to be rather based on the activity of specific endoproteinases (Kirchhoff et al., Food Chem **31** (1989), 295-311). This specific mixture of peptides and hydrophobic amino acids is deemed to represent cocoa-specific flavour precursors.

Until now several proteolytic enzyme activities have been investigated in cacao beans and studied for their putative role in the generation of cocoa flavour precursors during fermentation.

An aspartic endoproteinase activity, which is optimal at a very low pH (pH 3.5) and inhibited by pepstatin A has been identified. A polypeptide described to have this activity has been isolated and is described to consist of two peptides (29 and 13 kDa) which are deemed to be derived by self-digestion from a 42 kDa pro-peptide (Voigt et al., J. Plant Physiol. **145** (1995), 299-307). The enzyme cleaves protein substrates between hydrophobic amino acid residues to produce oligopeptides with hydrophobic amino acid residues at the ends (Voigt et al., Food Chem. **49** (1994), 173-180). The enzyme accumulates with the vicilin-class (7S) globulin during bean ripening. Its activity remains constant during the first days of germination and does not decrease before the onset of globulin degradation (Voigt et al., J. Plant Physiol. **145** (1995), 299-307).

Also a cysteine endoproteinase activity had been isolated which is optimal at a pH of about 5. This enzymatic activity is believed not to split native storage proteins in ungerminated seeds. Cysteine endoproteinase activity increases during the germination process when degradation of globular storage protein occurs. To date, no significant role for this enzyme in the generation of cocoa flavour has been reported (Biehl et al., Cocoa Research Conference, Salvador, Bahia, Brasil, 17-23 Nov. 1996).

Moreover, a carboxypeptidase activity has been identified which is inhibited by PMSF and thus belongs to the class of serine proteases. It is stable over a broad pH range with a maximum activity at pH 5.8. This enzyme does not degrade native proteins but preferentially splits hydrophobic amino acids from the carboxy-terminus of peptides (Bytof et al., Food Chem. **54** (1995), 15-21).

During the second step of cocoa flavour production – the roasting step – the oligopeptides and amino acids generated at the stage of fermentation are obviously subjected to a Maillard reaction with reducing sugars present in fermented beans eventually yielding substances responsible for the cocoa flavour as such.

In the art there have been many attempts to artificially produce cocoa flavour.

Cocoa-specific aroma has been obtained in experiments wherein acetone dry powder (AcDP) prepared from unfermented ripe cacao beans was subjected to autolysis at a pH of 5.2 followed by roasting in the presence of reducing sugars. It was conceived that under these conditions preferentially free hydrophobic amino acids and hydrophilic peptides should be generated and the peptide pattern thus obtained was found to be similar to that of extracts from fermented cacao beans. An analysis of free amino acids revealed that Leu, Ala, Phe and Val were the predominant amino acids liberated in fermented beans or autolysis (Voigt et al., Food Chem. **49** (1994), 173-180). In contrast to these findings, no cocoa-specific aroma could be detected when AcDP was subjected to autolysis at a pH of as low as 3.5 (optimum pH for the aspartic endoproteinase). Only few free amino acids were found to be released bu a large number of hydrophobic peptides were formed. When peptides obtained after the autolysis of AcDP at a pH of 3.5 were treated with carboxypeptidase A from porcine pancreas at pH 7.5, hydrophobic amino acids were preferentially released. The pattern of free amino acids and peptides was similar to that found in fermented cacao beans and to the proteolysis products obtained by autolysis of AcDP at pH 5.2. After roasting of the amino acids and peptides mixture as above, a cocoa aroma could be generated.

It has also been shown that, a synthetic mixture of free amino acids alone with a similar composition to that of the spectrum found in fermented beans, was incapable of generating cocoa aroma after roasting, indicating that both the peptides and the amino acids are important for this purpose (Voigt et al., Food Chem. **49** (1994), 173-180.

In view of the above data a hypothetical model for the generation, during fermentation, of the said mixture of peptides and amino acids, i. e. the cocoa flavour precursors, had been devised (Fig. 1), where in a first step peptides having a hydrophobic amino acid at their end, are formed from storage proteins, which peptides are then further degraded to smaller peptides and free amino acids. To produce the said peptides having C-terminal hydrophobic amino acids, an aspartic endoproteinase activity related to that mentioned above seems to be involved. Yet, for splitting off hydrophobic amino acids from peptides formed in the

preceding step the only known enzymatic activity, which might be considered in this respect, is that of a carboxypeptidase. However, such enzyme has not been isolated and studied in detail in cacao and it is therefore still questionable, which cacao enzyme might be responsible for the generation of hydrophobic amino acids required for cocoa flavour.

Though some aspects of cocoa flavour production have been elucidated so far there is still a need in the art to fully understand the processes involved, so that the manufacture of cocoa flavour may eventually be optimized.

A problem resolved by the present invention therefore resides in providing means for further elucidating the processes involved in the formation of cocoa-specific aroma precursors during the fermentation of cacao seeds, to improve the formation of cocoa flavour during processing and manufacturing and eventually providing means assisting in the artificial production of cocoa flavour.

This problem has been solved by providing a nucleotide sequence encoding a novel carboxypeptidase from cacao beans (termed *cacao CP-III*), which is identified by SEQ. ID. N°1, or functional derivatives thereof having a degree of homology of more than 80 %, preferably more than 90 % more preferably more than 95 %.

It will be appreciated by the skilled person that a gene encoding a specific polypeptide may differ from a given sequence according to the Wobble hypothesis, in that nucleotides are exchanged that do not lead to an alteration in the amino acid sequence. Yet, according to the present invention also nucleotide sequences shall be embraced, which exhibit a nucleotide exchange leading to an alteration of the amino acid sequence, such that the functionality of the resulting polypeptide is not essentially disturbed.

This nucleotide sequence may be used to synthesise a corresponding polypeptide by means of recombinant gene technology, in particular a polypeptide as identified by SEQ. ID. N°2.

As has been shown in a comparison with other carboxypeptidases from other plants the present enzyme does not show a substantial homology to any of the carboxypeptidases known so far. Since it is assumed, that cocoa may furthermore contain additional carboxypeptidases that might exhibit a higher homology to the carboxypeptidases known so far it must be considered as a surprising fact that this very enzyme has been detected.

For producing the polypeptide by recombinant means, the nucleotide of the present invention is included in an expression vector downstream of a suitable promoter and is subsequently incorporated into a suitable cell, which may be cultured to yield the polypeptide of interest. Suitable cells for expressing the present polypeptide include bacterial cells, such as e.g. *E.coli*, or yeast, insect, mammalian or plant cells.

The present DNA sequence may also be incorporated directly into the genome of the corresponding cell by techniques well known in the art, such as e.g. homologous recombination. Proceeding accordingly will provide a higher stability of the system and may include integration of a number of said DNA-sequences into a cell's genome.

The cells thus obtained may in consequence be utilised to produce the polypeptide in batch culture or using continuous procedures, with the resulting polypeptide being isolated according to conventional methods.

The recombinant carboxypeptidase obtained may be used for the manufacture of cocoa flavour. To this end, the enzyme described herein may be utilised in an artificial trial run, wherein a mixture of different proteins, such as cacao storage proteins, or protein hydrolysates of other resources, are subjected to enzymatic degradation by means of enzymes, known to be involved in proteolytic degradation to eventually assist in the production of flavour precursors. The enzyme may likewise also be utilised in the production of cocoa liquor, and in the manufacture of chocolate.

Yet, the present invention also provides plants, in particular cacao plants, comprising a

recombinant cell, containing one or more additional copies of the carboxypeptidase of the present invention. Such a cacao plant will produce beans, which will exhibit a modified degradation of storage proteins when subjected to the fermentation process, allowing a more rapid degradation or a pattern of hydrolysis that yields a higher level of cocoa flavour precursor, since a higher amount of carboxypeptidase will be present.

The carboxypeptidase of the present invention may also be used to produce other transgenic plants such as soybean and rice, producing seeds with this new protein modifying enzyme.

In the figures,

Fig. 1 shows a scheme illustrating a potential process for the proteolytic formation of cocoa-specific aroma;

Fig. 2 shows the cloning strategy used for the isolation of a cDNA encoding a carboxypeptidase from *Theobroma cacao*;

Fig. 3 shows a comparison of the hydrophilicity Plot-Kyte-Doolittle for the cacao CP-III sequence with Barley CP-MI, CP-MII and CP-MIII;

Fig. 4 shows a Northern blot analysis of cacao CP-III.

As described above, it was suggested that a carboxypeptidase could be involved in the production of cocoa flavour precursors during cacao fermentation. However it was not known in the art which cacao carboxypeptidase carried out this function considering that five classes of carboxypeptidases (Type I-V) have been identified in different plants by references to differences in substrate specificities, molecular weights and chromatographic profiles. Furthermore 50 sequences having homologies with serine carboxypeptidases exist in the completed *Arabidopsis* genome.

The following examples illustrate the invention further without limiting it thereto. In the examples the following abbreviations have been used.

PCR:	Polymerase Chain Reaction
RACE :	Rapid Amplification cDNA Ends
cDNA:	complementary deoxyribonucleic acid
mRNA:	messenger ribonucleic acid
DEPC:	Diethyl pyrocarbonate
3,4-DCI:	3,4-dichloroisocoumarin

Examples

Materials

Cacao (*Theobroma cacao* L.) seeds (male parent unknown) from ripe pods of clone ICS 95 were provided by Nestlé ex-R&D Center Quito (Ecuador). The seeds were taken from the pods immediately after arrival at Nestlé Research Center Tours (4-5 days after harvesting). The pulp and the seed coat were eliminated and the cotyledons were frozen in liquid nitrogen and stored at -80°C until use.

Preparation of mRNA

Total RNA was prepared using the following method. Two seeds were ground in liquid nitrogen to a fine powder and extraction was directly performed with a lysis buffer containing 25 mM Tris HCl pH8, 25 mM EDTA, 75 mM NaCl, 1% SDS and 1M β-mercaptoethanol. RNA was extracted with one volume of phenol/chloroform/isoamylalcohol (25/24/1) and centrifuged at 8000 rpm, 10 min at 4°C. The aqueous phase was extracted a second time with one volume of phenol/chloroform/isoamylalcohol (25/24/1). RNA was precipitated with 2M lithium chloride at 4°C overnight. The RNA pellet obtained after centrifugation was resuspended in DEPC treated water and a second precipitation with 3M sodium acetate pH 5.2 was performed in presence of two volumes of ethanol. The RNA pellet was washed with 70% ethanol and resuspended in DEPC treated water. Total RNA

was further purified using the Rneasy Mini kit from Qiagen®.

Cloning of a carboxypeptidase cDNA

Cloning strategy

A 1.5 kb 5' end fragment of a carboxypeptidase from cacao seed was amplified by RT-PCR using a degenerate oligonucleotide. Based on the sequence of this fragment, a primer was designed to amplify a 3'-end fragment. Finally, a full-length cDNA (*cacao CP-III*) was amplified using primers specific to both extremities (Fig. 2).

Primer design

A search for carboxypeptidase sequences in the GenBank database lead to the identification of several plant sequences. A multiple alignment of these sequences revealed the presence of conserved regions. The conserved sequence MVPMDQP located near the histidine catalytic site has been used to design a degenerate oligonucleotide in the antisense orientation: pCP2r (5'-GGYTGRTCCATNGGNACCAT).

Synthesis of cDNA

Total RNA (see above) was used to synthesise first strand 3' and 5' cDNAs with the SMART™ RACE cDNA Amplification Kit (Clontech, USA). Synthesis has been performed exactly as described in the kit instructions using 1 µg of total RNA and the Superscript™ II MMLV reverse transcriptase (Gibco BRL, USA). After synthesis, cDNAs were used directly for PCR or kept at -20°C.

5' RACE amplification

Specific cDNA amplification was performed with 2.5 µl of the first strand 5' cDNA in 50 µl buffer containing: 40 mM Tricine-KOH pH 8.7, 15 mM KOAc, 3.5 mM$Mg(OAc)_2$, 3.75 µg/ml BSA, 0.005% Tween-20, 0.005% Noninet-P40, 0.2 mM dNTP's, 14 pmoles of pCP2r primer, 5µl of 10X Universal primer Mix (UPM) and 1 µl 50X Advantage 2 polymerase Mix

(Clontech, USA). Amplification was performed in a Bio-med thermocycler 60 (B. Braun). A first denaturation step (94°C, 2 min) was followed by 35 cycles of denaturation (94°C, 1 min), primer annealing (55°C, 1.5 min) and extension (72°C, 2 min). The extension time was increased by 3 sec at each cycle. Amplification was ended by a final extension step (72°C, 10 min). The amplified fragment was cloned in pGEM®-T vector and sequenced.

3' RACE PCR

The sequence information obtained after the sequencing of the 5' end fragment was used to design a specific oligonucleotide pCP5 (5'-GCTTTTGCTGCCCGAGTCCACC), which was used for 3'-RACE amplification. 3'-RACE PCR was performed with 2.5 µl of SMART single strand 3' cDNA in 50 µl buffer containing 40 mM Tricine-KOH pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, 0.2 mM dNTP's, 10 pmoles of pCP5 primer, 10µl of 10X Universal primer Mix (UPM) and 1 µl 50X Advantage 2 polymerase Mix (Clontech, USA). Amplification was performed via touchdown PCR, in a Bio-med thermocycler 60 (B. Braun).

A first denaturation step (94°C, 1 min) was followed by:

- 5 cycles including denaturation at 94°C for 30 sec and annealing/extension at 72°C for 3 min
- 5 cycles including denaturation at 94°C for 30 sec and annealing/extension at 70°C for 30 sec and 72°C for 3 min
- 30 cycles including denaturation at 94°C for 30 sec and annealing/extension at 68°C for 30 sec and 72°C for 3 min.

The amplified fragment was cloned in pGEM®-T vector and sequenced.

Full length cDNA

The sequence information obtained after the sequencing of 5'-and 3'-RACE fragments was used to design two specific oligonucleotides.

pCP8: A sense primer (5'-CAAAGAGAAAAAGAAAAGATGGC)

pCP7r: A reverse primer (5'-CCCCAGAGCTTTACGATACGG).

PCR reaction was performed with 2.5 µl first strand cDNA in 50 µl buffer containing: 40 mM Tricine-KOH pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75µg/ml BSA, 0.005% Tween-20, 0.005% Noninet-P40, 0.2 mM dNTP's, 10 pmoles of pCP8 primer, 10 pmoles of pCP7r primer and 1 µl 50X Advantage 2 polymerase Mix (Clontech, USA). Amplification was performed in a Bio-med thermocycler 60 (B. Braun). A first denaturation step (94°C, 1 min) was followed by 35 cycles of denaturation (94°C, 30 sec), primer annealing (63°C, 1 min) and extension (72°C, 2 min). The extension time was increased by 3 sec at each cycle. Amplification was ended by a final extension step (72°C, 10 min). The amplified fragment was cloned in pGEM®-T Easy vector and sequenced.

Sequencing and analysis of DNA sequences

cDNA sequencing has been performed by Eurogentech (Belgium) and ESGS (France). Sequence analysis and comparison were performed with Lion's software bioScout, Lasergene software (DNAStar) and Genedoc programme.

The *cacao CP-III* cDNA sequence is 1768 bp long. A putative initiation start codon was assigned by comparison with other carboxypeptidase sequences. It is located 25 bp from the 5' end. The open reading frame is broken by a stop codon (TGA) at position 1549, followed by a putative polyadenylation signal (TATAAA) at position 1725.

Cacao CP-III encodes a 508 amino acid type III carboxypeptidase C with a predicted molecular weight of 56 kDa and a pI of 5.04. The catalytic amino acids are present at position Ser^{228}, Asp^{416} and His^{473}. Hydrophilicity analysis (Fig. 3) reveals that *cacao CP-III* encodes a hydrophilic protein with a very hydrophobic N-terminal end, indicating the presence of a signal peptide.

Northern blot analysis

Total RNA samples were separated on 1.5 % agarose gel containing 6% formaldehyde (Fig.4). After electrophoresis, RNA was blotted onto nylon membranes (Appligene) and hybridized with ^{32}P-labeled cacao CP-III probe at 65°C in 250 mM Na-phosphate buffer pH

7.2, 6.6% SDS, 1 mM EDTA and 1% BSA. Cacao CP-III cDNA fragment was amplified by PCR using pCP8 and pCP7R primers and labelled by the random priming procedure (*redi*prime™ II, Amersham Pharmacia Biotech). Membranes were washed three times at 65°C for 30 min in 2XSSC, 0.1%SDS, in 1XSSC, 0.1% SDS and in 0.5XSSC, 0.1%SDS.

Claims

1. A nucleotide sequence coding for a carboxypeptidase and having a sequence as identified by SEQ. ID. No 1 or functional variants thereof having a degree of homology of more than 90 %.

2. A polypeptide encoded by a nucleotide according to claim 1.

3. The polypeptide according to claim 2, which is identified by SEQ. ID. No 2.

4. A vector containing a DNA sequence according to claim 1.

5. A cell containing a recombinant DNA sequence according to claims 1 and 4.

6. The cell according to claim 5, which is a bacterial, a yeast, an insect, a mammalian or a plant cell, preferably a cacao cell.

7. Transgenic plants, containing a cell according to claim 6.

8. Use of a nucleotide according to claim 1 for the synthesis of a carboxypeptidase.

9. Use of a polypeptide according to any of the claims 2 or 3 for the manufacture of cocoa flavour.

10. Use of a polypeptide according to any of the claims 2 or 3 for the manufacture of cocoa liquor and chocolate.

11. Use of a polypeptide according to any of the claims 2 or 3 for hydrolyzing proteins.

12. The use according to claim 7, wherein the proteins are derived from food material.

13. A process for producing cocoa flavour comprising subjecting material to yield cocoa flavour precursors to an enzymatic degradation, involving the use of a carboxypeptidase according to any of the claims 2 or 3.

14. A product containing cocoa flavour, obtained according to the method of claim 9.